REMARKS

The Official action mailed 25 June 2008, has been received and its contents carefully noted. Claims 37-46 were rejected. Claims 37, 40, 44 and 46 have been amended and claims 47-57 have been added. Support may be found in the Specification and the claims as originally filed. See, for example, the Specification as originally filed on page 43, paragraph 171. The Specification is amended to change certain boldface type to regular font. No statutory new matter has been added. Therefore, reconsideration and entry of the claims as amended are respectfully requested.

Information Disclosure Statement

The Examiner indicated that two references in the IDS were not considered because the dates were missing.

Applicants respectfully submit that the two "references" which were struck thru by the Examiner are continued information for the references which were provided on the last line of the previous page, i.e. page breaks split the information for the two references such that the information for each is found on the bottom of one page and the top of the next page.

Therefore, Applicants respectfully submit that the Examiner has properly considered Schmid & Adler (1998) and DiDonato (1996) and request that the record be clarified.

Rejection under 35 U.S.C. 112, first and second paragraphs - Derivative

The Examiner rejected claims 37-46 under 35 U.S.C. 112, first paragraph, as lacking written description support and second paragraph as being indefinite. Specifically, the Examiner deemed that the Specification does not provide support for "derivatives" of rottlerin. Applicants have canceled references to a "derivative" in the claims.

Therefore, Applicants respectfully assert that the claims, as amended, have adequate written description support and are clear and definite. Thus, the rejections under 35 U.S.C. 112, first and second paragraphs, should properly be withdrawn.

Rejection under 35 U.S.C. 112, second paragraph

The Examiner rejected claims 39-40 under 35 U.S.C. 112, second paragraph, as being

indefinite as references to "the polyphenol" were unclear.

Applicants respectfully urge that the claims, as amended, are clear and definite and the rejection under 35 U.S.C. 112, second paragraph, should properly be withdrawn.

Rejection under 35 U.S.C. 103(a)

The Examiner rejected claims 37-46 under 35 U.S.C. 103(a) as being unpatentable over Schwartz (US 5,821,072), Gschwendt and Mouria. Specifically, the Examiner deemed that it would have been obvious to administer rottlerin which is a PKC inhibitor to treat pancreatic cancer since Gschwendt discloses that a specific PKC inhibitor may be used to treat pancreatic tumors. The Examiner then cited Mouria for administering genistein to treat pancreatic cancer.

Applicants respectfully submit that one of ordinary skill in the art would not have a reasonable expectation of success in using rottlerin or a compound as set forth in claim 47 could be used to treat pancreatic cancer. Specifically, merely because rottlerin is a PKC inhibitor and other PKC inhibitors are known to potentiate apoptosis in tumor cells is not enough to make the leap to the assumption that rottlerin will likely be successful in treating pancreatic cancer.

The reason this leap can not be made is because "[b]oth the activation and inhibition of PKC have been linked with the induction of apoptosis" as explained in the review by Mackay and Twelves (enclosed). See page 2, 1st col., 2nd full paragraph, see also Podar Abstract (enclosed). It is noted that Mackay and Twelves evidence this fact with scientific evidence which was in the art prior to and around the time of the instant application. Thus, one of ordinary skill in the art would not have had a reasonable expectation that rottlerin, a PKC inhibitor, could be used to successfully treat pancreatic cancer. Instead, because of the duality of PKC activation and inhibition, one of ordinary skill in the art would not have a reasonable expectation of success until rottlerin was actually tested in pancreatic cancer models. Since the cited art do not provide any disclosure or evidence that rottlerin was actually tested in pancreatic cancer models, there is no reasonable expectation of success.

Thus, Applicants respectfully submit that one skilled in the art would not have been motivated to administer rottlerin or a compound according to claim 47 with a reasonable likelihood of successfully treating or inhibiting pancreatic cancer or pancreatitis.

Therefore, the claimed invention is unobvious and the rejection under 35 U.S.C. 103(a) should properly be withdrawn.

Request for Interview

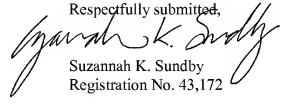
Either a telephonic or an in-person interview is respectfully requested should there be any remaining issues.

CONCLUSION

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Therefore, it is respectfully requested that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. It is believed that a full and complete response has been made to the outstanding Official action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

It is not believed that extensions of time are required, beyond those that may otherwise be provided for in accompanying documents. However, in the event that additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. 1.136(a), and any fees required therefor are hereby authorized to be charged to **Deposit Account No. 02-4300**, Attorney Docket No. **034044.021CIP1**.

Respectfully submitted,



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The therapeutic role of targeting protein kinase C in solid and hematologic malignancies.

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The protein kinase C (PKC) family, the most prominent target of tumor-promoting phorbol esters, is functionally linked to cell differentiation, growth, survival, migration and tumorigenesis and so mediates tumor cell proliferation, survival, multidrug resistance, invasion, metastasis and tumor angiogenesis. Therefore, targeting PKC isozymes may represent an attractive target for novel anticancer therapies. Recent preclinical and clinical studies using the macrocyclic bisindolylmaleimide enzastaurin or the N-benzylstaurosporine midostaurin demonstrate promising activity of PKC inhibitors in a variety of tumors, including diffuse large B-cell lymphoma, multiple myeloma and Waldenstroem's macroglobulinemia. However, our knowledge of PKCs in tumorigenesis is still only partial and each PKC isoform may contribute to tumorigenesis in a distinct way. Specifically, PKC isoforms have vastly different roles, which vary depending on expression levels of organ and tissue distribution, cell type, intracellular localization, protein-protein and lipid-protein interactions and the biologic environment. Although PKC activation generally positively affects tumor cell growth, motility, invasion and metastasis, recent reports show that many PKCs can also have negative effects. Therefore, it is necessary to further dissect the relative contribution of PKC isozymes in the development and progression of specific tumors in order to identify therapeutic opportunities, using either PKC inhibitors or PKC activators.

PMID: 17922632 [PubMed - indexed for MEDLINE]

OPINION

Targeting the protein kinase C family: are we there yet?

Helen J. Mackay and Christopher J. Twelves

Abstract | Protein kinase C (PKC) comprises a family of serine/threonine kinases that are involved in the transduction of signals for cell proliferation, differentiation, apoptosis and angiogenesis. Unsurprisingly, disruption of PKC regulation is implicated in tumorigenesis and drug resistance. PKC function is complex in this context owing to the differing roles of individual isozymes within the cell and across tumour types. Therapeutically targeting PKC isozymes is not new; however, with many of the early PKC inhibitor cytotoxic drug combinations being discarded at the phase II level, and recent phase III studies in non-small-cell lung cancer proving negative, what's going wrong?

Intracellular serine/threonine kinases mediate many signalling pathways important for downstream signal transduction following the activation of receptor tyrosine kinases. The protein kinase C (PKC) family consists of at least 12 such kinases with distinct and in some cases opposing roles in cell proliferation, differentiation, apoptosis and angiogenesis[1,2]. Early observations that PKC isozymes are activated by tumour-promoting phorbol esters[3] suggested a key role for PKC in tumour promotion and progression leading to PKC being considered as a target for cancer therapy[4].

PKC regulation and function

PKC was originally identified as a phospholipid and calcium-dependent protein kinase[5]. The subsequent classification of the isoforms is based on structural and activational characteristics. There are nine PKC genes that code for isozymes classified into 3 groups (FIG. 1): classical or conventional PKCs (cPKCs: PKCα, PKCβI, PKCβII and PKCγ), which are calcium dependent and activated by both phosphotidylserine (PS) and diacylglycerol (DAG); novel PKCs (nPKCs: PKCδ, PKCε, PKCη and PKCθ), which are calcium independent and regulated by DAG and PS; and atypical PKCs (aPKCs: PKCζ and PKCλ), which are calcium independent and do not require DAG for activation, although PS can regulate their activity[6,7]. Many of these kinases show overlapping substrate specificities *in vitro*. Consistent with their different biological functions, PKC isoforms differ in their structure, tissue distribution, subcellular localization, mode of activation and substrate specificity. The activation of PKC isozymes results in changes in their subcellular location following translocation to specific anchoring proteins. Cell-specific isoform functions may be conferred by differences in subcellular localization[8]. Furthermore, compartmentalization in different subcellular locations contributes to differential activation[10,11]. These factors raised the possibility of developing PKC isozyme-specific inhibitors with the potential for targeting specific intracellular pathways in tumour cells, and possibly even in specific tumour cell types.

The phospholipid DAG has a central role in the activation of PKC by causing an increase in the affinity of PKCs for cell membranes accompanied by PKC activation and the release of an inhibitory substrate (a pseudo-substrate) to which the inactive enzyme binds[6]. Activated PKC then phosphorylates and activates a range of kinases. Signals that stimulate G-protein-coupled receptors, receptor tyrosine kinases and non-receptor protein tyrosine kinases can all cause the production of DAG[6,7]. Several PKC isotypes are activated independently in a redundant manner through the phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3K) pathways[6]. There is some evidence to suggest that there are functional differences between PKCs activated through different pathways[12]. The downstream events following PKC activation are little understood, although the MEK–ERK (mitogen activated protein kinase kinase–extracellular signal-regulated kinase) pathway is thought to have an important role[13,14] (FIG. 2). There is also evidence to support the involvement of PKC in the PI3K–Akt pathway[15]. PKC isozymes probably form part of the multiprotein complexes that facilitate cellular signal transduction.

The complexities of PKC and cancer

The function of PKC in cancer is complex, primarily because much of the data indicate that the isozymes subtly regulate many pathways involved in cellular transformation. Overall, increased PKC levels have been associated with malignant transformation in several cell lines including breast[17], lung[18] and gastric carcinomas[16]. *In vivo*, however, the relationship is less clear. For example, the expression of PKCβ in colon tumours has been shown to be increased, the same or decreased compared with normal epithelium. Not surprisingly, this relationship becomes even more complex when considered at the level of individual isozymes. PKCβ has been implicated in proliferation and differentiation, with the two splice variants of the *PKCβ* gene appearing to have opposing cellular functions[9,21,22]. Overexpression of PKCβII in colonic epithelium results in hyperproliferation and increased susceptibility to carcinogenesis, probably through the cyclooxygenase 2 (COX2) and transforming growth factor-β (TGFβ) signalling pathways. The expression of PKCβII increases significantly early on in carcinogenesis[21,23], whereas PKCβI is associated with differentiation and is downregulated in colonic tumours[23]. Furthermore, in rat intestinal epithelial cells grown in culture and in animals, PKCβII regulates its own expression through the mitogen activated protein kinase (MAPK)–ERK-dependent signalling pathway. In breast cancer, PKC activity is increased compared with normal tissue[24], and is associated with oestrogen receptor negativity[26]. However, the overexpression of PKCα and PKCβI is reported to induce a less aggressive phenotype[27].

Adding to this complexity is the fact that PKC is activated in response to many signals — it functions in many pathways that are important in tumorigenesis. For example, vascular endothelial growth factor (VEGF) acts through VEGF receptor 2 (VEGFR2; also known as kinase insert domain-containing receptor) and subsequent PLCγ tyrosine phosphorylation to promote the activation of PKCβ in endothelial cells[28]. PKC has also been implicated in the epidermal growth factor receptor (EGFR)[29] pathway, which is disrupted in various human tumours.

The PKC family also has a role in cellular adhesion, and is therefore important for cancer cell invasion mediated by integrin binding, the activation of matrix

metalloproteinases and the expression of extracellular matrix proteins. Inhibition of PKC activity produces a reduction in invasiveness paralleled by the inhibition of cell motility in some cell lines[90,91].

The interplay between PKC isozymes is complex, making it hard to predict the impact of inhibiting one isozyme in the clinic. For example, stable overexpression of PKCα in a breast cancer cell line increases the expression of PKCβ accompanied by decreased levels of PKCη and PKCδ[92].

PKC and apoptosis. Both the activation and inhibition of PKC have been linked with the induction of apoptosis. PKCα and PKCβII generally seem to be anti-apoptotic[93,94]. PKCα phosphorylates the anti-apoptotic protein BCL2, potentiating its anti-apoptotic function[95]. However, PKCδ can be pro- or anti-apoptotic depending on the cell type or signal received[96,97]. PKCδ increases the sensitivity of human glioma cells to chemotherapy[98], but promotes survival and resistance to cytotoxic agents (including paclitaxel and cisplatin) in non-small-cell lung cancer (NSCLC)[99]. Another mechanism by which the PKC family influences apoptosis and cellular proliferation is through the PI3K–Akt pathway. Phosphotidylinositol kinase 1 (PDK1), an effector kinase downstream of PI3K, phosphorylates and activates PKC[16]. Furthermore PKCs α, β and η directly phosphorylate Akt[40,67,68]. Moreover, both PKCδ[34] and Akt[45] can phosphorylate glycogen synthase kinase 3β (GSK3β), a serine/threonine kinase involved in metabolism, development and apoptosis. Therefore, cross-talk between the PI3K and PKC pathways might influence apoptosis. Interestingly, VEGF is regulated at the post-transcriptional level through the activation of the Akt pathway, and this might explain the anti-angiogenic effects of some PKC inhibitors (see below)[46].

PKC and drug resistance. Perhaps one of the most controversial roles of PKC is its involvement in cytotoxic drug resistance. An association between PKC activation and increased multidrug resistance (MDR) was first reported in 1988 [REF. 47]. Later studies suggested this was caused by increased PKCα expression, although in some cases it appeared to be due to increased expression of PKCβ or γ[48]. Thus, PKC was viewed as a potential target for overcoming drug resistance. Interest in this area diminished following the results of two major studies that ruled out PKC-catalysed phosphorylation of P-glycoprotein (also known as MDR1) as a mechanism that regulates drug efflux[49,50].

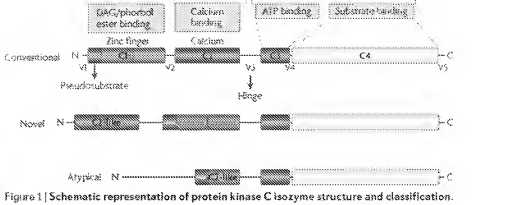

Figure 1 | **Schematic representation of protein kinase C isozyme structure and classification.** PKC has 4 conserved domains (C1–4): C1 comprises 1 or 2 cysteine-rich motifs that form the diacylglycerol (DAG) and phorbol ester binding site. C2 contains the recognition site for acidic lipids and in some enzymes the calcium binding site. C3 and C4 form the ATP and substrate binding lobes of the catalytic site. The C2 domain of novel PKCs lacks amino acids to bind calcium. Atypical PKCs have only 1 cysteine-rich motif, and phorbol ester binding has not been detected.

However, this conclusion is based on the assumption that direct phosphorylation is the sole mechanism for PKC regulation of MDR. PKC isozymes have subsequently been associated with the transcription of ATP-binding cassette transporters such as MRP1 and LRP (in addition to MDR1)[51]. Therefore, selective isozyme inhibition might still have a role in the reversal of MDR.

Why is targeting PKC such a problem?

Many of the difficulties in designing an effective, therapeutic strategy for inhibiting PKCs arise from the limited data that are available from patient samples. Hence, it is not clear whether data obtained in cell lines translate into the clinic. Many consider this to be the biggest shortfall in the PKC clinical discovery field. Initially, immunohistochemistry was used to measure 'global' PKC levels in clinical samples[52,53]. However, as the individual PKC isozymes and their differing cellular functions were identified it became apparent that this approach was too crude.

PKC isozymes and immunohistochemistry. Attempts have been made in many different tumour types to correlate the immunochemistry of individual isozymes to known prognostic features. In the main, however, study numbers have been small and, therefore, little information is available on the prognostic value of the PKC isozymes, and nothing is known about their role in predicting response to conventional therapy. For example, in normal breast and breast cancer samples, PKCα expression shows a progressive reduction in staining intensity from normal breast to invasive ductal carcinoma (IDC). These changes appear to occur at a stage between epithelial hyperplasia and ductal carcinoma *in situ* (DCIS)[54]. The pattern of cytoplasmic staining suggests that PKCα activity is altered in dividing or abnormal cells. The authors of these findings raise the possibility that an alteration in the subcellular localization of PKCα leads to changes in the desmosomal adhesive state of the cells. This potentially leads to a loss in cell–cell adhesion and a transition from a normal to a malignant phenotype.

In bladder cancer, PKC isoform expression assessed by western immunoblot differentially alters as a function of tumour grade. The expression of PKCβ and δ decreases with increasing tumour grade, whereas the levels of PKCα, and ζ increase. These findings suggest a potential role for the PKC family in the transformation of the human bladder epithelium[54]. These opposite changes seen in the levels of the isozymes occur with *in vitro* data, suggesting that individual isozymes can act antagonistically. In this context, these data show that the opposing behaviour of PKC isozymes can be seen even within members of the same PKC subfamily. Therefore, it appears that the differential expression pattern cannot solely be ascribed to different activation mechanisms.

In ovarian cancer, a reduction in the expression of PKCα occurs with increasing grade of malignancy[54], this contrasts with the overexpression of PKCα reported in endometrial[55] and prostate[56] carcinomas. PKCδ was not expressed in ovarian tumours, and PKCι was expressed in a subset of tumours and was associated with a poor outcome[56].

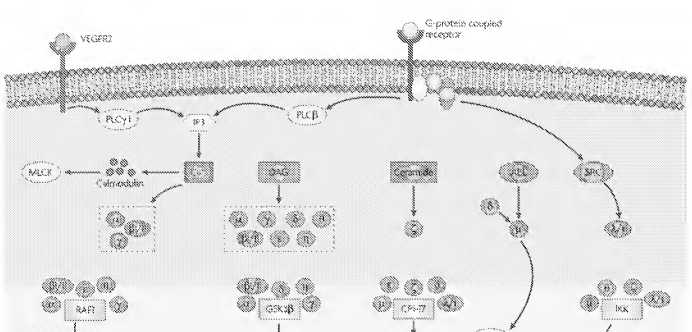

Figure 2 | **Proposed effects of protein kinase C activation.** The protein kinase C family influence many cellular pathways. Some isozymes can be activated by several different pathways, such as release of calcium (Ca^{2+}), or production of diacylglycerol (DAG). Others are activated by one pathway, such as ceramide. Many of the isozymes display overlapping substrate specificities *in vitro*, and may interact to control a number of signalling pathways that regulate cell-cycle control, proliferation, apoptosis, cellular adhesion and metastasis. CPI-17, protein kinase C potentiated inhibitor protein; ERK, extracellular signal-regulated kinase; GSK3β, glycogen synthase kinase 3β; HDAC7 histone deacetylase 7; IKK, IκB kinase complex; MEK, mitogen activated protein kinase kinase; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PKC, protein kinase C; PLC phospholipase C; VEGFR2, vascular endothelial growth factor receptor 2.

However, there are problems inherent in using immunochemistry as a tool to investigate the PKC family. First there are technical issues with the use of antibodies; for example, anti-PKCβ antibodies also detect PKCγ. Second, immunochemistry is not particularly useful for the accurate quantification or identification of the subcellular localization of a protein. And third, a positive immunochemistry result does not reflect the functional status of the protein, or give an insight into the multiprotein signalling complexes of which PKC isozymes form a part. Further work using techniques such as real-time PCR to better define the level of expression and immunofluorescence or immunogold labelling to identify sub-cellular localization is required. Moreover, other techniques such as array-based comparative genomic hybridization (CGH) might also prove to be useful. For example, high resolution CGH identified the upregulation of PKCι in epithelial ovarian cancer samples that correlated with tumour stage and grade. These data were validated using reverse transcription PCR (RT-PCR), and the overexpression of PKCι was confirmed by tissue array and western blot. By combining these results with cell line studies the authors of this work suggest that PKCι might be acting as an oncogene, cooperating with mutant Ras[36]. The role of PKCι as a biomarker for aggressive disease needs to be investigated further in future studies.

Some of the technical issues surrounding antibody use are being addressed; for example, Lahn *et al.*[35], compared 2 commercially available antibodies in 63 NSCLC samples and concluded that the monoclonal was superior to the polyclonal antibody. They also correlated PKCα protein expression with mRNA expression together with gene array data in an attempt to interpret PKCα-associated gene-expression profiles in 40 NSCLC specimens. PKCα expression was higher in adenocarcinoma than in squamous cell carcinoma, a finding confirmed by the gene-expression array data. However, the small sample size meant that it was not possible to establish an association between PKCα overexpression and survival. Furthermore, by analysing PKCα phosphorylation in 29 stage I or II NSCLC specimens, the authors concluded that when PKCα is detected by immunochemistry or quantitive PCR it is likely to be the phosphorylated (and presumably active) protein. The amount of phosphorylation was variable and unrelated to the degree of staining observed using immunochemistry[35]. This study was small, but illustrates the challenges inherent in trying to rationally select patients who might benefit from a PKC isozyme targeted approach.

Ultimately the success or failure of any therapeutic approach targeting the PKC pathway rests on the identification of valid PKC isozyme targets. We need larger scale studies to determine how isozymes affect prognosis and predict response to conventional treatment. The papers by Lahn *et al.*[35,36] show how different approaches can be combined to elucidate the role of PKCα

Table 1 | **Protein kinase C inhibitors**

Drug	Class	Route of administration	Specificity/selectivity	Comments	Refs
PMA (TPA)	Phorbol ester	Intravenous	Non-specific	PKC activator; in a phase I trial in haematological malignancy	116
Staurosporine	Indolocarbazole	Intravenous	Poor specificity, also inhibits other serine/threonine kinases and tyrosine kinases	Preclinical	11
PKC412 (midostaurin)	Indolocarbazole	Oral	PKCs α, β, γ, δ, ε, η, also inhibits tyrosine kinase pathways	Potentiates treatment with doxorubicin or vinblastine; in phase II trials	11,63,72
UCN01	Indolocarbazole	Intravenous	cPKCs > nPKCs; check point kinase inhibitor	Potentiates treatment with cisplatin, mitomycin C, camptothecin or 5FU; in phase II trials	119–125
Go6976	Indolocarbazole	Intravenous	cPKCs > nPKCs		126
Bryostatin 1	Macrocyclic lactone	Intravenous	Activator of cPKC and nPKCs, in presence of activating ligands acts as an antagonist	Potentiates treatment with cytosine arabinoside, paclitaxel, tamoxifen or vincristine	88,98–100
Tamoxifen	Nonsteroidal anti-oestrogen	Oral	PKCs α, β, γ non-selective		127
Bisindolylmaleimide (LY333531)	Indolocarbazole	Oral	PKCβ	Used in the treatment of diabetic retinopathy	128
LY317615 (enzastaurin); Ro31-8220; Ro32-0432; GF109203X	Indolocarbazole	Oral	PKCβ	Potentiates treatment with gemcitabine, 5FU, cisplatin or radiotherapy	78,80–83
ISIS3521 (aprinocarsen)	Antisense oligonucleotide; 18-mer phosphorothioate oligodeoxynucleotide	Intravenous	PKC^{α}	In phase II and phase III trials	119–116
ISIS9606	Antisense oligonucleotide; 19-mer phosphorothioate oligodeoxynucleotide	Intravenous	PKCα	Not developed further in the clinic	

A list of the main protein kinase inhibitors. PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; 5FU, 5-fluorouracil.

in NSCLC. It may be that we need combinations of techniques to be able to identify and validate approaches that can be developed into screening tests for individual patient populations.

Protein kinase inhibitors

Given their many cellular roles, PKC isozymes are undoubtedly attractive targets for therapeutic intervention. However, the very factors that make them attractive targets also pose significant problems in designing an inhibitory strategy. The complexity of their interactions and the many secondary messenger systems involved coupled with their cellular and tissue-specific variability, renders selective drug action difficult. The principal agents under investigation are listed in Table 1.

ATP-competitive compounds

Staurosporine, identified 20 years ago as an antiproliferative agent, is a potent inhibitor of PKC. It probably acts as a competitive inhibitor by binding to the conserved ATP-binding sites on PKC. Although its specificity for PKC and its isozymes is poor, staurosporine has served as a lead compound from which many other PKC inhibitors have been developed[11].

Midostaurin. Midostaurin (also known as PKC412 or *N*-benzoylstaurosporine), similar to UCN01 (7-hydroxystaurosporine), exhibits improved selectivity for PKC ATP binding sites, but shows modest isozyme specificity. In addition to nPKCs and cPKCs, midostaurin inhibits other tyrosine kinase pathways, including VEGFR2, fms-related tyrosine kinase 3 (FLT3), platelet-derived growth factor receptor (PDGFR) and KIT kinases[63].

In preclinical studies midostaurin showed a broad range of anti-tumour activity, synergizing with conventional cytotoxic agents without overt toxicity and reversing p-glycoprotein-mediated multidrug resistance *in vitro* and *in vivo*[64,65].

Midostaurin was well tolerated in a phase I study, with the main toxicities being nausea, vomiting, diarrhoea and fatigue. A formal maximum tolerated dose (MTD) was not defined[66]. Similar to UCN01, midostaurin has a longer half-life than would be predicted from preclinical studies owing to altered gastrointestinal absorption and plasma protein binding (in particular, binding to α-1-acid glycoprotein) in patients with cancer.

PKC is involved in signalling pathways upstream of tumour necrosis factor-α (TNFα) and can induce release of TNFα and the induction of interleukin 6 (IL6)[66,67]. cPKCs, in addition to other kinases targeted by midostaurin (including SRC and protein kinase A), are involved in the early stages of T-cell activation, and midostaurin interferes with T-cell activation and inhibits TNFα[66,68]. In the phase I study the release of both TNFα and IL6 from phytohaemagglutinin-stimulated whole blood cells isolated from treated patients was significantly

inhibited in a time- and dose-dependent manner. In addition, the downregulation of ERK2, which is downstream from PKC [illegible], was demonstrated across the 50–300 mg a day dose range[76]. The recommended phase II dose of 150 mg a day was determined by the increased prevalence of symptomatic toxicities taken together with the pharmacodynamic data.

PKCα expression is increased in some melanoma cell lines[74], and midostaurin inhibits PKC activity in melanoma cells and delays the development of lung metastasis in mice[73]. Therefore, a phase II trial was instigated in patients with malignant melanoma, and patients with accessible tumours were biopsied to examine drug efficacy. However, midostaurin failed to show anti-tumour activity. There was a large inter-patient variability in midostaurin plasma concentration owing to plasma protein binding. It was, therefore, perhaps not surprising that the results showed an inconsistent and variable inhibition of PKC activity in tumour biopsies, and no modulation of multidrug resistance[74].

These studies highlight the problems inherent in using this class of drug. The high and variable plasma binding meant that it was a challenge to reach drug levels *in vivo* that are known to inhibit PKC. In common with other agents the selection and use of pharmacodynamic endpoints is problematic. It is not clear whether immunomodulatory effects, such as changes in TNFα and IL6 production, reflect anticancer activity, or even whether these changes are a result of PKC inhibition. Furthermore, it is not clear what degree of inhibition of the chosen target (ERK2 or PKC activity) is representative of efficient drug activity. In tumour samples taken from three patients in the phase II melanoma study, the expression of PKC isozymes that are known to be relatively insensitive to inhibition by midostaurin (notably PKCδ) was reported. Therefore, patient selection might be key if we are to see activity from these agents.

Midostaurin is showing promising results in combination with imatinib mesylate in patients with gastrointestinal stromal tumours[75]. However, this probably reflects the inhibitory effect of midostaurin on PDGFRα, PDGFRβ and KIT rather than an effect on PKC. Midostaurin is also under investigation in haematological malignancies, and has been proposed as an immunomodulating agent for patients with conditions such as psoriasis and inflammatory bowel diseases that express high levels of TNFα[74].

Enzastaurin. Enzastaurin (LY317615) disrupts the intrinsic phosphotransferase activity of PKCβ. At low concentrations it is relatively specific for PKCβ, but at higher concentrations it inhibits other PKC isozymes[68]. PKCβ, as discussed previously, is part of the VEGF signalling pathway that activates the MAPK and ERK cascade, which subsequently affects endothelial proliferation. As a result, enzastaurin was predominantly developed as an anti-angiogenic agent. This activity was confirmed in cell line and human tumour xenograft-bearing mice[66,77]. Interestingly, ovarian cell line data suggested that enzastaurin might be effective in taxane-resistant but not cisplatin-resistant ovarian tumours[78]. Enzastaurin also directly affects tumour growth by inducing apoptosis and suppressing cellular proliferation through the PI3K–Akt pathway. Enzastaurin induces apoptosis and suppresses proliferation in a range of cultured tumour cell lines through the Akt pathway, suppressing the phosphorylation of GSK3β, ribosomal protein S6 and Akt[77]. As VEGF is regulated at the post-transcriptional level by the Akt pathway, reductions in VEGF expression observed following the administration of enzastaurin might be explained by this mechanism[66]. In human colon and glioblastoma xenograft-bearing mice, oral dosing with enzastaurin suppresses the phosphorylation of GSK3β to a comparable degree in both tumour tissue and peripheral blood mononuclear cells (PBMCs), suggesting that PBMCs might be a useful surrogate tissue in future studies[77].

In a phase I study, enzastaurin was well tolerated with no MTD identified. The investigators pre-selected a target mean steady state concentration of enzastaurin based on the free fraction of drug that produced 90% (IC_{90}) inhibition of PKCβ *in vitro* and on plasma protein binding. The recommended oral daily dose, based on this pharmacokinetic target and observed toxicities, was 525 mg. The main toxicities were fatigue and gastrointestinal, and 21 patients (47%) achieved stable disease, although there were no objective responses[79]. However, questions still remain about the optimal dose for this agent. The target IC_{90} was based on *in vitro*, not *in vivo* studies, and there is no data to suggest that this dose will inhibit PKCβ in tumours. Furthermore, there was significant inter-patient variability, with 5 out of 12 patients having levels below the target threshold[79].

Green *et al.* have recently published the development and validation of a biomarker assay assessing intracellular phosphoprotein signalling in PBMCs[80]. They have developed this assay as a surrogate for enzastaurin activity in patients, but it remains to be seen if and how this will affect the development of this drug.

The PKCβ and PI3K–Akt pathways are frequently activated in glioblastoma, making this an attractive tumour type in which to further investigate enzastaurin. Reports from a phase II trial in patients with recurrent high-grade gliomas were promising, with objective responses observed in 14 out of 79 evaluable patients[81]. However, a randomized phase III study was recently stopped by E. Lilly on the recommendation of the External Data Monitoring Committee. A planned interim analysis suggested it would not meet its primary end point (progression-free survival).

A phase III study in patients with diffuse large B-cell lymphoma is currently underway (PRELUDE; Preventing Relapse in Lymphoma Using Daily Enzastaurin). Patients who are in remission following first-line therapy are randomized to maintenance therapy of enzastaurin or placebo. Additional phase II studies have been instigated in patients with colon, ovarian or pancreatic carcinomas as well as patients with NSCLC. Combination studies with conventional cytotoxic agents have also been investigated[66,81]. Preliminary reports from a phase I study combining enzastaurin with gemcitabine and cisplatin look promising, with 3 out of 17 patients achieving a partial response[82]. Further combination studies are listed in TABLE 2.

Bryostatins

The bryostatins are a family of at least 20 naturally occurring macrocyclic lactones derived from the marine bryozoan *Bugula neritina*[83]. The prototype compound for this class of drugs is bryostatin 1.

Bryostatin 1. Bryostatin 1 is a potent modulator of PKC activation[84–87]. Short-term exposure of tumour cells to bryostatin 1 results in cPKC and nPKC activation and translocation to the nuclear membrane[88]. Conversely, prolonged exposure results in membrane depletion of PKC and decreased PKC activity[85]. In addition, bryostatin 1 downregulates some PKC isozymes, notably PKCα, through the proteasomal degradation of the enzyme[89]. In preclinical models bryostatin 1 inhibits cell growth, angiogenesis and promotes differentiation and apoptosis. The basis for the divergent activities of bryostatin 1 derive from differential isoform activation (PKCs α, δ and ε)[86] or nuclear translocation (PKCβ)[87].

Table 2 | **Combination studies with standard cancer therapeutics and protein kinase C inhibitors**

Combination	Phase	Comments	Refs
Gemcitabine and cisplatin +/- aprinocarsen	Phase III (NSCLC)	No benefit; N = 670, median overall survival 10 month versus 10.4 months	115
Paclitaxel and carboplatin +/- aprinocarsen	Phase III (NSCLC)	No benefit	116
Gemcitabine, carboplatin and aprinocarsen	Phase II (NSCLC)	Modest activity; median overall survival 8.3 months; toxicity: 78% G3/4 thrombocytopenia	114
Gemcitabine, enzastaurin and cisplatin	Phase I	Findings only reported in published abstract to date	82
Enzastaurin and capecitabine	Phase I	No objective response, stable disease in 5 patients	83
Enzastaurin and pemetrexed	Phase I	Findings only reported in published abstract to date	129
L-threo-dihydrosphingosine (Safingol) and cisplatin	Phase I	Some activity seen in two patients with refractory adrenocortical cancer	130
UCN01 and topotecan	Phase I and II (ovarian)	Inactive	131
UCN-01 and 5FU	Phase I	No objective response, seven patients with stable disease	132
Paclitaxel and bryostatin 1 (pancreatic cancer)	Phase II	Inactive; first stage criteria not met; one minor response in 14 evaluable patients	103
Paclitaxel and bryostatin 1 (hormone refractory prostate cancer)	Phase II	Inactive; no objective response; PSA response in 3/17 patients; study terminated at first stage	104
Bryostatin 1 and interleukin 2	Phase II (RCC)	Inactive	105
Bryostatin 1 and gemcitabine	Phase I	DLT myalgia, myelosuppression; partial response in 2 out of 36 patients (breast and colon cancer)	96
CCI 779 and bryostatin 1	Phase I	Evidence of activity in 23% of patients; response in one patient with RCC; stable disease in five patients (four RCC and one sarcoma)	106
Midastaurin and Imatinib	Phase I and II (gastrointestinal stromal tumour)	Some activity likely to be related to PDGFR inhibition	73

DLT, dose-limiting toxicity; 5FU, 5-fluorouracil; L-threo-dihydrosphingosine, a saturated optical isomer of naturally occurring sphingosine that inhibits PKC; NSCLC, Non-small-cell lung cancer; PDGFR, platelet-derived growth factor receptor; PSA, prostate specific antigen; RCC, renal cell carcinoma

but whether the same mechanisms are active in every tumour type is not clear. Furthermore, bryostatin 1 activates effector cells of the immune system and stimulates cytokine production. It induces IL2 receptor expression on $CD4^+$ and $CD8^+$ T lymphocytes, and when combined with a calcium ionophore, can stimulate the production of IL2 and the proliferation of T lymphocytes. *In vitro* bryostatin 1 promotes the development of cytotoxic T lymphocytes (CTLs) and decreases the amount of IL2 needed for the development of CTLs[87,88]. Whether the immunomodulatory effects of bryostatin 1 contributes to its cytotoxicity is not clear.

In HL-60 chronic lymphocytic leukaemia cell lines bryostatin 1 promotes the induction of apoptosis by cytosine arabinoside[89]. Similar results in cell lines were reported with vincristine, cisplatin and gemcitabine[90–92]. As with cytosine arabinoside, the best results were obtained when bryostatin 1 treatment preceded the cytotoxic agent. However, in a tumour-bearing mouse model, pretreatment with bryostatin 1 decreases the activity of paclitaxel (a G2–M-phase acting drug), whereas the reverse sequence increases efficacy compared with single agent paclitaxel. This probably reflects bryostatin 1-induced inhibition of $p34^{CDC2}$ kinase activity (not a PKC-related effect) that is accompanied by a decrease in the expression of cyclin B1, which would result in the inhibition of proliferation and so limit the efficacy of paclitaxel[93].

Based on the theory that prolonged exposure downregulates PKC activity, bryostatin 1 has been widely investigated in a series of phase I trials with intravenous infusion times varying from 1 to 24 hours[94–103]. The dose-limiting toxicity (DLT) in all studies was myalgia (muscle pain, the aetiology of which remains unclear), and localized phlebitis (inflammation of the veins) at the infusion site was a feature of the shorter infusion durations. Significant increases in plasma concentrations of TNFα and IL6, chosen as markers of PKC inhibitory activity, were observed when 50 μg per m^2 of bryostatin 1 was given as a weekly 1 hour infusion for 3 weeks out of 4 [94,95]. A further study of a weekly 24 hour infusion of bryostatin 1 showed significant changes in the levels of activated PKC in PBMCs during the infusion[96]. The previously reported increase of IL6 and TNFα was not confirmed in this study. However, an increase in IL2 induced proliferative response in PBMCs and increased lymphokine-activated killer cell activity were shown. Once again, how this correlates with cytotoxic efficacy is not clear.

Bryostatin 1 showed some evidence of anti-tumour activity in phase I trials, but single-agent phase II studies in a wide range of tumour types, including melanoma[104] and colorectal cancer[98], were disappointing. Phase II studies combining bryostatin 1 with other cytotoxic agents have yet to show efficacy[103,104,105,106]. Given the reported upregulation of IL2 by PKC, a phase II study was conducted combining IL2 with bryostatin 1 in patients with renal cell carcinoma. Although it was well tolerated, the addition of bryostatin 1 did not appear to improve response rates, nor was there an effect on T-cell expansion, activation or cytokine production[105].

Recently, an interesting approach was reported in abstract form stating that the combination of bryostatin 1 with the mammalian target of rapamycin (mTOR) inhibitor CCI779 resulted in anti-tumour activity in one patient (a 23% reduction in tumour volume) and stable disease in a further five patients. The combination was well tolerated, and there was evidence of inhibition of the

mTOR pathway[106]. Bryostatin 1 is also being investigated in haematological malignancies in combination with rituximab in patients with non-Hodgkin lymphoma and chronic lymphocytic leukaemia.

Given the pleiotropic effects that bryostatins have, it is not clear what are the most promising targets to measure in terms of predicting anticancer activity in any given tumour type. Currently there are no data on the predictive value of individual PKC isoenzymes in terms of bryostatin efficacy.

Antisense oligonucleotides

In order to achieve greater isozyme specificity, antisense oligonucleotides or peptide fragments have been developed to either inhibit or promote the translocation of PKC isoforms to specific anchoring proteins[107].

Aprinocarsen. Aprinocarsen (ISIS 3521), is a 20-mer oligonucleotide that hybridizes to the 3′ untranslated region of *PKCα* mRNA, and inhibits its expression through RNAse-mediated cleavage[107]. The phosphorothioate structure conveys increased resistance to exonuclease and endonuclease-mediated degradation, resulting in a stable heteroduplex with its target mRNA. Preclinical studies with this agent included evaluation in a range of cell lines and xenograft models[107,108,109]. Administration of aprinocarsen resulted in sequence-specific and concentration-dependent inhibition of the mRNA for *PKCα*, and reduced the production of PKCα protein[109].

Aprinocarsen is rapidly taken up into tissues followed by a prolonged elimination half-life. Continuous infusion was associated with greater uptake into tissues, prolonged inhibition of *PKCα* mRNA and reduced plasma concentrations. As a result, protracted infusions were selected as the best schedule for developing this agent. In phase I studies the main toxicities were fatigue, nausea, vomiting, fever and chills, and thrombocytopenia. Dose escalation was discontinued owing to fatigue and thrombocytopenia at 3 mg per kg (body weight). Furthermore, as some oligonucleotide backbones cause activation of the complement cascade with an associated risk of death at high doses in primates, investigators took this into consideration when selecting the dose of 2mg per kg (body weight) a day given as a continuous infusion[108]. Anti-tumour activity was shown in non-Hodgkin lymphoma and ovarian carcinoma in a phase I study[110], but a phase II study in breast cancer failed to show any activity[111], and no clinical benefit was demonstrated in a phase II study in patients with recurrent high-grade astrocytomas[112]. Phase I and II studies of aprinocarsen in combination with carboplatin and paclitaxel in NSCLC achieved a 42% response rate, suggesting potentiation of chemotherapy activity[113]. A further phase I and II trial of aprinocarsen combined with gemcitabine and cisplatin in NSCLC showed a 38% response rate and 55% stable disease[114]. Despite these encouraging results, two randomized phase III studies in NSCLC failed to show a benefit from the addition of aprinocarsen to gemcitabine and cisplatin or to paclitaxel and carboplatin[115,116]. However, aprinocrasen was infused over 14 days, starting either day 1 or 3 of 21 day cycles in the phase III studies, unlike the continuous infusions used in the phase II studies. Taken together these results suggest that, although well tolerated, aprinocarsen fails to increase survival or other efficacy measures in patients with advanced NSCLC treated with chemotherapy. However, there is no validated predictive biomarker for response to aprinocarsen, and patients were not screened for PKCα levels. Complement levels were measured in some studies, and although potentially valuable in predicting toxicity, it is unclear how these relate to the efficacy of this agent.

So, where next?

The PKC family represents an interesting and challenging target for the development of new therapeutic agents. However, so far results in the clinic have been disappointing. So does the protein kinase C family remain a viable target for new drug development? As with many targeted therapeutic approaches there are several reasons why the first generation of agents have had limited success. First, most of the current PKC inhibitors are relatively non-specific in their actions. As we have discussed, given the complexity of the functions and interactions of the PKC isozymes it is perhaps not surprising that agents targeting multiple isozymes give mixed results. Developing agents that target a single isoform, different activating pathways, specific membrane interactions or events further down the signalling pathways are underway and may be more successful. Second, our understanding of the relative importance of PKC isoforms outside cell line models is limited. We need to better understand the roles, interactions and significance of the PKC family within our patients. Once again, the complexity of the PKC family and their differing roles within different tumours indicates that 'one size will not fit all'. Third, in developing these agents, the identification of cellular targets and development of validated predictive biomarkers will be key if we are to achieve success. In the aprinocarsen studies patients were not screened for levels of PKCα expression and, in common with many of the PKC studies, no validated biomarker predictive of response to the drug was available[115,116]. Despite the negative results we still do not know if there was a sub-set of patients who benefited from this agent. Fourth, this is an evolving field in cell biology, even when specific agents such as aprinocarsen have been used, emerging data may invalidate the target in a given tumour type. For example, preclinical studies suggest that, unlike other isoforms, PKCα is not a driving force in NSCLC cellular proliferation[98]. Once again, this information is crucial in selecting patient populations in which to test these drugs.

Fifth, in studies that examine the combination of PKC inhibitors with conventional cytotoxics, the optimal combination and the sequence in which these drugs can be used needs to be carefully evaluated, bearing in mind that particular combinations might only work in particular tumour types. Combination studies with bryostatin 1 show the importance of sequencing when designing clinical trials. Combining specific PKC isozyme inhibitors with other biological agents represents a further area of interest. The combination of an 'upstream–downstream' approach to pathway inhibition might be more effective than either agent alone. In preclinical studies PKCζ inactivation increases the growth inhibition mediated by an EGFR inhibitor in a head and neck cancer cell line[68]. In addition, the receptor tyrosine kinase inhibitor and anti-angiogenic agent Sorafenib only partially inhibits the growth of glioma cell lines at clinically achievable concentrations; however, the addition of rotterlin, a PKCδ inhibitor, increases the antiproliferative effect of Sorefanib[117]. Furthermore, potentially targeting VEGF and protein kinase-β might increase the anti-angiogenesis actions of both agents.

Conclusion

Successes with other signal transduction inhibitors, such as imatinib, have shown that the development of the right agent for the right target can work. However, we have much to learn, both in the clinic and the laboratory, about targeting the PKC family, but by using integrated information and intelligent trial design we will get there.

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Competing interests statement
The authors declare no competing financial interests.

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